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INTRODUCTION

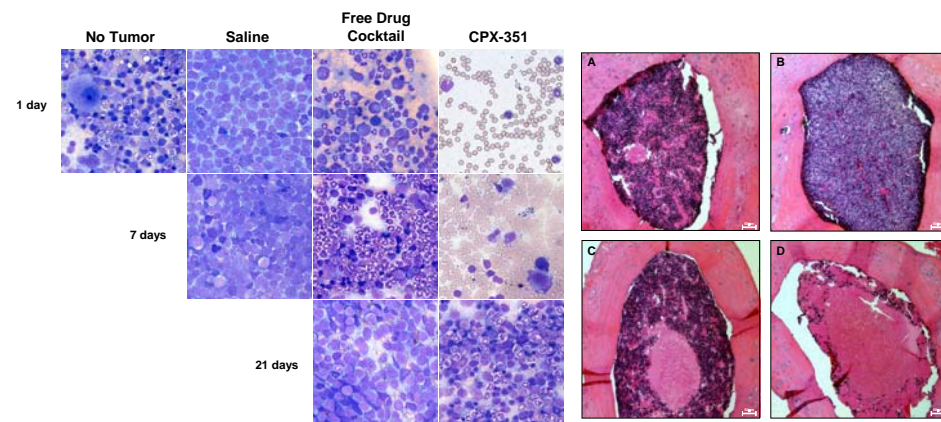
Combination chemotherapy consisting of cytarabine and an anthracycline (typically daunorubicin or idarubicin) have been one of the most effective induction treatments for acute myelogenous leukemia (AML) for over three decades. This therapy results in complete response rates of 50-60%, although most patients ultimately relapse and only 20-30% will be cured of their disease. CPX-351 is a newly developed liposomal formulation of cytarabine:daunorubicin co-encapsulated at a synergistic 5:1 molar drug ratio. This formulation was shown to have superior therapeutic activity compared to unencapsulated cytarabine:daunorubicin in various murine and human leukemia tumor models (Tardi et al., Leukemia Research 33:129-139, 2009). In the present study, we utilized the CCRF-CEM bone marrow engrafted human lymphocytic leukemia xenograft model to elucidate the pharmacodynamic basis for the superior efficacy of CPX-351 over free drug cocktail.

Key Observations

The administration of CPX-351 to CCRF-CEM tumor bearing mice resulted in the complete ablation of leukemic cells in the bone marrow for multiple weeks, whereas the saline-based drug cocktail induced only transient leukemia suppression. When administered to healthy mice, the unencapsulated cytarabine:daunorubicin cocktail and CPX-351 provided comparable bone marrow cell suppression after intravenous treatment at MTD, indicating CPX-351 tumor selectivity. Examination of cytarabine and daunorubicin concentrations in the bone marrow revealed dramatically enhanced CPX-351 drug levels relative to the free drug cocktail. Confocal fluorescence microscopy of leukemia cells exposed to CPX-351 in vitro revealed that CPX-351 liposomes were taken up into cytoplasmic vacuoles and subsequently released their drug contents intracellularly. We believe that a combination of enhanced drug delivery to the bone marrow and the selective intracellular delivery of the synergistic cytarabine:daunorubicin ratio contribute to the overall superiority of CPX-351 over conventional free drug cocktail.

RESULTS

1 Bone marrow smears and cross-sections reveal marked superiority in antileukemic activity for CPX-351 treatment in the CCRF-CEM human leukemia xenograft model

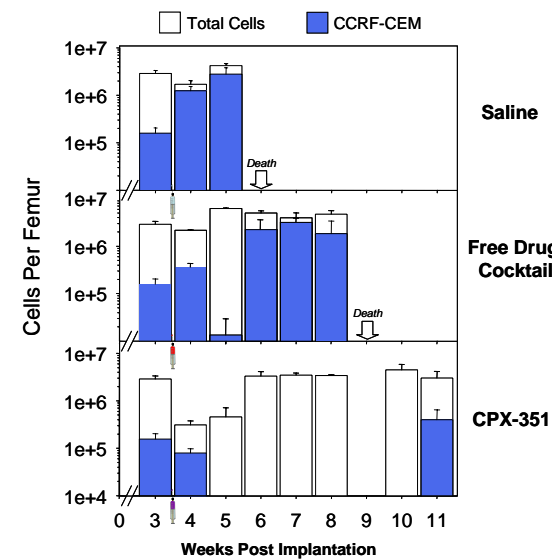


Left panel: Bone marrow smears of tumor free marrow or CCRF-CEM engrafted Rag2-M mice following treatment with either saline, MTD of cytarabine:daunorubicin free drug cocktail (300:4.5 mg/kg) or MTD of CPX-351 (10 mg/kg cytarabine and 4.4 mg/kg daunorubicin) administered on days 21, 24 and 27. Smears were prepared on days 1, 7 and 21 following the last dose.

Right panel: Femur cross-sections from tumor free marrow (A) or CCRF-CEM engrafted Rag2-M mice following i.v. treatment with either saline (B), MTD of cytarabine:daunorubicin free drug cocktail (C) or MTD of CPX-351 (D) administered on days 21, 24 and 27. Cross sections were prepared one day following the last dose.

Methods: CCRF-CEM human acute T-cell lymphoblastic leukemia cells were inoculated i.v. (1×10^7) into the lateral tail vein of female Rag2-M mice. Tumor bone marrow engraftment was observed in all untreated mice with death occurring by day 42 (52 of 52 mice). Bone marrow smears and cross-sections were prepared by cutting femurs from female Rag2-M mice in half using bone rongeurs. Marrow was removed using a dissecting needle and smeared onto a microscope slide for air drying, fixing and staining using the May-Grunwald-Giemsa method. Formalin-fixed femurs were sent to Wax-It Histology Services for resin-embedding, cross-sectioning and H&E staining.

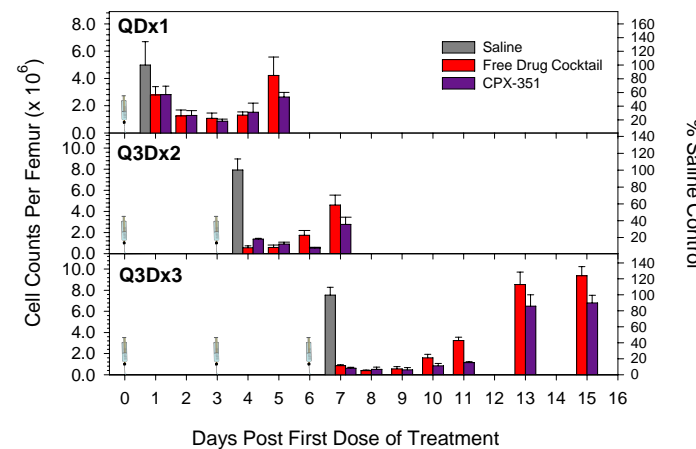
2 CPX-351 causes ablation of bone marrow engrafted leukemia cells followed by normal bone marrow recovery. In contrast, free drug cocktail induces incomplete and transient leukemia suppression.



CCRF-CEM tumor engrafted mice were treated i.v. with either saline, MTD of the cytarabine:daunorubicin free drug cocktail (300:4.5 mg/kg) or MTD of CPX-351 (cytarabine:daunorubicin, 10:4.4 mg/kg) on days 21, 24 and 27. Prevalence of the leukemic cell population was monitored weekly throughout the study period.

Methods: Flow cytometry was performed to quantify normal bone marrow and CCRF-CEM tumor cell populations. Briefly, femoral bone marrows from CCRF-CEM engrafted mice were isolated and incubated with FITC-conjugated anti-human CD4 monoclonal antibodies. Cell debris and residual RBC were gated out of the analysis while FITC fluorescence was detected by excitation/emission wavelengths of 488/525 nm. Each time point represents the average \pm standard error of 3 or more femurs with 50,000 cells analyzed per femur.

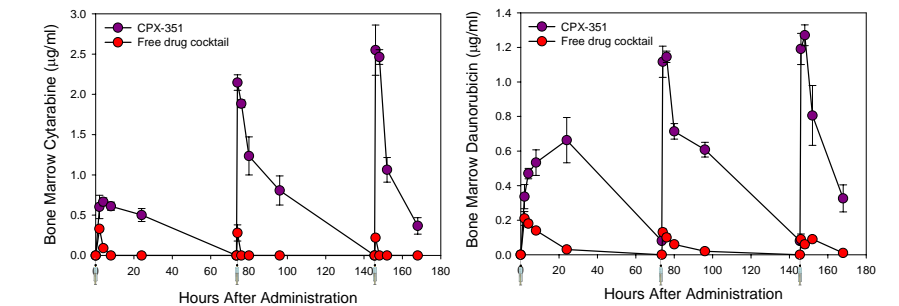
3 Comparable suppression of mouse femoral normal bone marrow cells induced by free drug cocktail and CPX-351 indicates selectivity of CPX-351 for leukemia cells



Total bone marrow cellularity in healthy BDF-1 mice was determined following treatment with either saline, cytarabine:daunorubicin free drug cocktail or CPX-351 at their respective MTD. Reductions and recovery of total bone marrow cell numbers over time (days) following one, two or three doses on a Q3D schedule are presented above.

Methods: Flow cytometry was performed to quantify the effect of treatment schedule on suppression and recovery of normal bone marrow cell populations. Briefly, femoral bone marrows from healthy BDF-1 mice were isolated following the indicated treatment schedule. Cell debris and residual RBC were gated out of the analysis and cell concentration determined by flow cytometry. Total cell counts were based on total volumes.

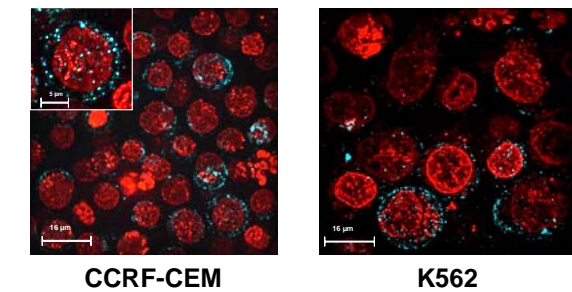
4 Bone marrow drug levels increase following multiple dosing with CPX-351 but not with free drug cocktail



CCRF-CEM tumor engrafted mice were treated with either MTD of the cytarabine:daunorubicin free drug cocktail (300:4.5 mg/kg) or the MTD of CPX-351 (cytarabine:daunorubicin, 10:4.4 mg/kg) on days 21, 24 and 27 post CCRF-CEM tumor cell implantation. Bone marrow levels of cytarabine and daunorubicin were determined throughout the dosing schedule.

Methods: Freshly harvested bone marrow samples were extracted with methanol or acidified methanol, with gemcitabine and idarubicin as internal standard. Cytarabine was quantified using ion-exchange HPLC on a Phenomenex SCX column, with 10 mM ammonium formate (pH 3.0) as mobile phase at 1.5 mL/min and UV detection at 278 nm. Daunorubicin was quantified using reverse phase HPLC on a Phenomenex Luna C18(2) column, with 67.5:32.5 (v/v) 25 mM ammonium acetate (pH 4.8) : acetonitrile as mobile phase at 1 mL/min and fluorescence detection (Ex = 480 nm Em = 560 nm).

5 CPX-351 is taken up intact by lymphocytic and myelocytic human leukemia cells and releases drug intracellularly



Leukemic cells were incubated with fluorescent DiD-lipid labeled CPX-351 for 30 minutes. Cells were washed 3x with cold HBSS and fluorescence visualized using a Quorum Wave FX confocal system. The DiD-labeled liposomes are colored cyan and daunorubicin fluorescence is red. The intracellular daunorubicin fluorescence represents the release of drug from the liposomes because encapsulated daunorubicin in CPX-351 is self-quenched and is not visible by fluorescence microscopy.

CONCLUSIONS

- CPX-351 is superior to the cytarabine:daunorubicin free drug cocktail in ablating leukemic cells from bone marrow as determined by flow cytometry and histological analysis of the bone marrows.
- CPX-351 and the cytarabine:daunorubicin free drug cocktail treatment provided comparable cellular suppression and recovery in normal bone marrow.
- Bone marrow drug levels remained comparable after every dose of saline based cocktail but CPX-351 accumulation was enhanced with multiple dosing, leading to significantly elevated drug concentrations.
- Confocal microscopy of CCRF-CEM and K562 cells in vitro revealed the intracellular delivery and nuclear accumulation of daunorubicin.
- Increased bone marrow accumulation and intracellular delivery of synergistic cytarabine:daunorubicin ratios may enhance the efficacy of CPX-351.